The temporal requirement for endothelin receptor-B signalling during neural crest development

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Endothelin receptor B (EDNRB) is a G-protein-coupled receptor with seven transmembrane domains which is required for the development of melanocytes and enteric neurons. Mice that are homozygous for a null mutation in the *Ednrb* gene are almost completely white and die as juveniles from megacolon. To determine when EDNRB signalling is required during embryogenesis, we have exploited the tetracycline-inducible system to generate strains of mice in which the endogenous *Ednrb* locus is under the control of the tetracycline-dependant transactivators tTa or rtTA. By using this system to express *Ednrb* at different stages of embryogenesis, we have determined that EDNRB is required during a restricted period of neural crest development between embryonic days 10 and 12.5. Moreover, our results imply that EDNRB is required for the migration of both melanoblasts and enteric neuroblasts.

During embryogenesis, neural crest cells arise from the dorsal end of the closing neural tube. These cells are pluripotent, giving rise to a wide range of cell types including neurons and glia of the peripheral nervous system, craniofacial bones and cartilage, smooth muscle cells and melanocytes. Upon their emergence from the neural tube, neural crest cells undergo extensive proliferation as they migrate along distinct pathways to destinations where they differentiate into specific lineages[1,2].

Murine coat colour mutants have been useful for identifying genes that are important for the development of neural crest-derived melanocytes[3,4]. The *Ednrb* gene, encoding a G-protein-coupled receptor with seven transmembrane domains, is deleted in the spontaneous mutant *piebald-lethal* ($Ednrb^{s\text{-}l}$)[5]. $Ednrb^{s\text{-}l}$ mutants are almost completely white, except for occasional pigmented areas in the head and rump, and die as juveniles from megacolon, a condition resulting from the inability of neural crest-derived enteric neurons to colonize the distal colon. The ligand for EDNRB is the 21-amino-acid peptide endothelin-3 (EDN3), and mutations in the *Edn3* gene underlie another classic coat colour mutant, *lethal spotting* ($Edn3^{ls}$). These mice closely resemble $Ednrb^{s\text{-}l}$ mutants, and exhibit severe melanocyte and enteric neuron defects[6]. Mutations in both *EDNRB* and *EDN3* have been described in humans with Hirschsprung's disease and Waardenburg syndrome, who have enteric neuron and pigmentation defects, respectively[7,8].

Previous results have indicated that EDNRB signalling may be required at multiple stages of melanocyte development. First, the expression of the melanoblast-specific marker tyrosinase-related protein (TRP-2 or DCT) in $Ednrb^{s\text{-}l}$ mice is greatly reduced at embryonic day (E) 10.5, implying that EDNRB signalling is required at or before the time of melanoblast proliferation and migration[9]. Second, *in vitro* studies have implicated EDN3 as both a potent mitogen for melanoblast precursor cells, implying an early requirement and a differentiation factor, indicating a possible role for the ligand after birth[10,11]. Finally, it has been suggested, on the basis of pigmentation differences in $Ednrb^{s\text{-}l}$ and $Edn3^{ls}$ mice, that EDNRB may be required during the intermediate stages of epidermal proliferation at E12.5–13.5 (ref. 12).

For mammalian developmental biologists, the ability to express a gene in a spatially and temporally controlled manner to study its function in the organism has been elusive. In lower eukaryotes, inducible systems such as heat shock and GAL4 are routinely used to achieve conditional expression of genes[13,14]. Among the many inducible systems that have been used in mammals[15], the bacterial based tetracycline (tet)-inducible system offers many advantages[16,17]. These include the low basal expression and high inducibility of the reporter gene, the overall nontoxic effect of tet, and the ability of tet and its derivatives to cross the placenta without affecting the development of embryos[18,19].

To date, however, the use of the tet-inducible system in the context of transgenes has met with limited success[18–22]. The proper spatial expression of the tet-dependent transactivators, tTA and rtTA, has been difficult to accomplish owing to the limited availability of well behaved promoters. In addition, transgene integration is random and, depending upon the site, can result in low or mosaic expression of the activators, as well as leaky expression of the responder genes[23].

To circumvent these problems and to ensure that the *Ednrb* gene is regulated in a spatially appropriate manner, we have integrated the tet-inducible system into the endogenous *Ednrb* locus. We show that this approach leads to tet-induced *Ednrb* expression that can fully rescue the null phenotype and to tet-dependent repression of *Ednrb* expression that recapitulates the null phenotype. By manipulating the expression of the gene during gestation with the tet derivative doxycycline (dox), we have identified a restricted time during which EDNRB signalling is required for the development of both melanoblasts and enteric neurons.

Incorporating the tet system into the *Ednrb* locus

To ensure that the tTA and rtTA proteins were expressed in all *Ednrb*-expressing lineages, we constructed targeting vectors in which the majority of the *Ednrb* first exon and 230 base pairs (bp) of the first intron were replaced with the *tTA* or *rtTA* coding region linked to the rabbit β-globin intron/polyadenylation sequences (Fig. 1). The first exon encodes the amino-terminal extracellular domain and the first transmembrane domain of EDNRB; deletion of any one of the membrane-spanning domains of G-protein-coupled seven-transmembrane receptors results in a null allele[24]. A neomycin-resistance gene flanked by two *loxP* sites was placed downstream of the *tTA* or *rtTA* cassettes (Fig. 1a, b). This strategy puts the expression of the transactivator proteins under the control of the endogenous *Ednrb* promoter.

For the responder targeting vector, the region from 550-bp upstream of the putative *Ednrb* promoter to 240-bp downstream of the exon 1/intron 1 junction was replaced with a cassette

containing a tet-responsive promoter that directs the expression of *Ednrb* complementary DNA (*tetO–Ednrb*). This vector contains, in addition to 8.7 kilobases (kb) of *Ednrb* genomic DNA, a hygromycin resistance gene flanked by two *loxP* sites (Fig. 1c). By integrating *tetO–Ednrb* into the *Ednrb* locus, we hoped that the cDNA would be in a chromatin state that would be accessible to the inducer proteins in the appropriate cell types.

The inducer and responder targeting vectors were separately introduced into embryonic stem cells by electroporation, and cell lines that had undergone homologous recombination were identified by Southern analysis with probes that lie outside the targeting vector (Fig. 1a–c; and data not shown). Mice carrying the modified *Ednrb* loci were generated. Initial analysis of the transactivator lines raised the possibility that the presence of the *neo* cassette inhibited expression of *tTA* and *rtTA* from the *Ednrb* promoter. To alleviate this problem, male offspring of the inducer lines were each mated to *EIIa-cre* transgenic females, which express cre recombinase at high levels in the fertilized egg[25], to establish $Ednrb^{tTA}$ and $Ednrb^{rtTA}$ lines without neo.

Ednrb is regulated *in utero* by dox

To test the efficacy of the tet system, we generated compound heterozygous $Ednrb^{rtTA}/Ednrb^{tetO}$ offspring, identified with specific polymerase chain reaction primers designed to distinguish the *Ednrb*, $Ednrb^{rtTA}$ and $Ednrb^{tetO}$ alleles (data not shown). rtTA has been engineered to activate the *tetO* promoter only in the presence of dox (Fig. 1d). As shown in Fig. 1e, two $Ednrb^{rtTA}/Ednrb^{tetO}$ neonates that developed without dox being given to the mothers were almost completely white, and eventually died of megacolon. Thus the targeted *Ednrb* alleles had disrupted normal *Ednrb* expression, and no leaky expression of *tetO–Ednrb* was evident. *Ednrb* is highly sensitive to gene dosage, and expression of messenger RNA at 12% of wild-type levels leads to pigmentation in the coat[26]. When dox-containing water was provided to the females for the entire pregnancy, the compound heterozygous offspring exhibited no melanocyte or enteric neuron defects, and no other abnormalities were observed (Fig. 1f).

The complementary tTA system, in which dox represses transactivation by tTA, was also effective; compound heterozygous $Ednrb^{tTA}/Ednrb^{tetO}$ mice that developed in the absence of dox were fully pigmented and viable, whereas those raised in the presence of dox displayed the *piebald-lethal* phenotype (Fig. 1d; and data not shown). These results confirmed that dox administered to pregnant females crossed the placenta and could activate or repress the expression of *Ednrb* cDNA from the tet-inducible promoter in the embryos.

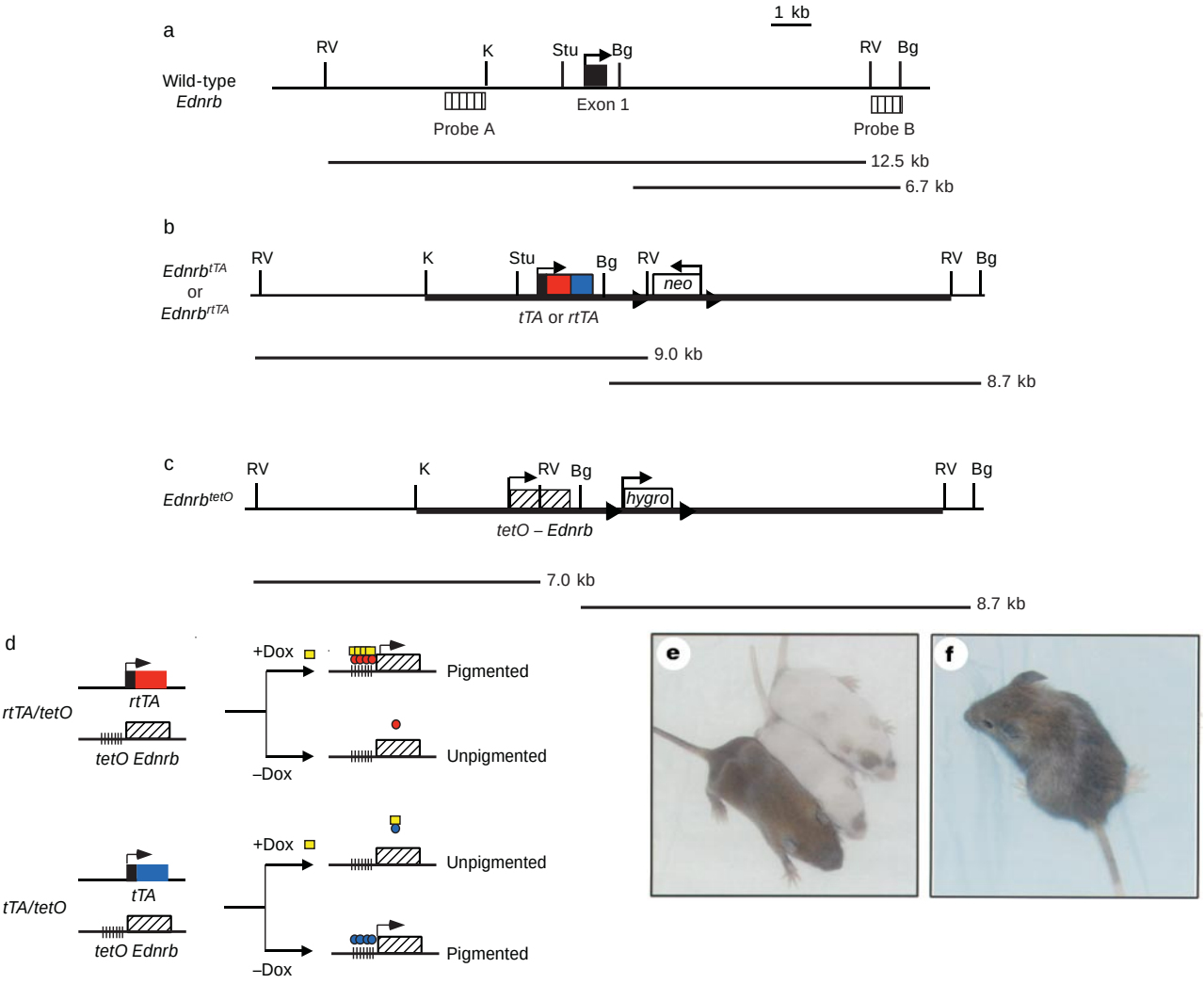

Figure 1 Establishing tet-responsive alleles of *Ednrb*. **a**, The region surrounding the *Ednrb* first exon. **b**, **c**, The targeted *Ednrb* locus containing the transactivators tTA or rtTA or the tet-responsive *Ednrb* cDNA gene. *LoxP* sites are indicated by filled triangles. The thick lines show the DNA included in the targeting vectors. RV, *Eco*RV; K, *Kpn*I; Stu, *Stu*I; Bg, *Bgl*II. **d**, The strategy for regulating *tetO–Ednrb* expression by dox. The *rtTA* and *tTA* genes and their protein products are indicated in red and blue, respectively. Dox is indicated by a yellow square. **e**, **f**, Unpigmented *tetO–Ednrb/rtTA* compound heterozygotes that developed in the absence of dox (**e**) and a pigmented compound heterozygote that developed in the presence of dox (**f**).

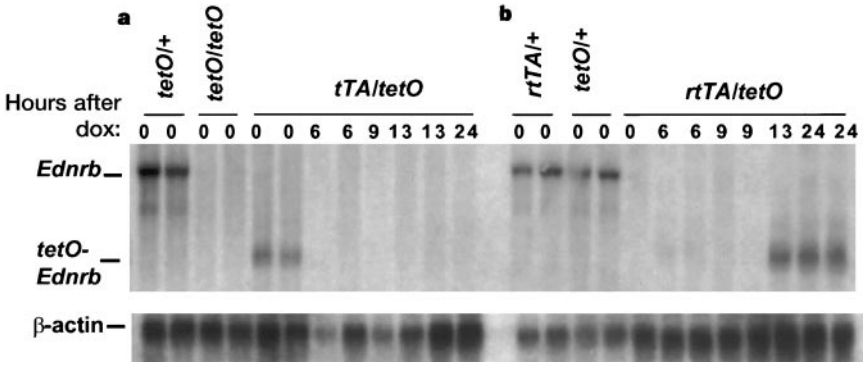

Figure 2 Kinetics of *Ednrb* regulation by dox. **a**, Repression of *tetO–Ednrb* mRNA by dox. $Ednrb^{tet0}$/+ females were mated to $Ednrb^{tTA}/Ednrb^{tet0}$ males and given dox at E11.5 by oral gavage. Embryos were obtained at the times indicated, and RNA was isolated and analysed by northern blotting with an *Ednrb* cDNA probe. The blots were stripped and re-probed with a β-actin cDNA. **b**, Induction of *tetO–Ednrb* mRNA by dox. $Ednrb^{tet0}$/+ females were crossed to $Ednrb^{rtTA}/Ednrb^{tet0}$ males, and treated as in **a**.

In vivo kinetics of *Ednrb* activation and repression

To determine the kinetics of *tetO–Ednrb* activation and repression in response to dox *in utero*, mRNA was extracted from individual $Ednrb^{tTA}/Ednrb^{tetO}$ E11.5 embryos. In the absence of dox, $Ednrb^{tTA}/Ednrb^{tetO}$ embryos expressed only the 1.8-kb *tetO–Ednrb* mRNA, whereas $Ednrb^{tetO}$/+ littermates expressed only the 4.5-kb endogenous *Ednrb* mRNA, and $Ednrb^{tetO}/Ednrb^{tetO}$ embryos did not express either *Ednrb* RNA (Fig. 2a). These results confirmed at the molecular level that all targeting events created *Ednrb* null alleles and that no leaky expression occurred from the *tetO* promoter.

Dox was administered to pregnant females by oral gavage on the morning of E11.5, and the animals were maintained on dox water for various times. $Ednrb^{tTA}/Ednrb^{tetO}$ embryos were identified and examined for *Ednrb* RNA expression. By 6 hours after dox administration, the shortest time examined, the expression of $Ednrb^{tetO}$ was undetectable by northern analysis (Fig. 2a).

$Ednrb^{rtTA}/Ednrb^{tetO}$ embryos did not express any *Ednrb* RNA in the absence of dox. After dox administered by oral gavage, *tetO–Ednrb* mRNA was highly induced within 13 hours (Fig. 2b). The difference in the response kinetics of tTA and rtTA has been described by *in vitro* studies, and implies that the tTA system responds to dox at lower concentrations than the rtTA system[27]. Despite the kinetic differences, our experiments clearly show that *in utero* activation and repression of *Ednrb* by maternal transmission of dox is rapid and efficient.

The earliest requirement for *Ednrb* function

The latest time when *Ednrb* activation by rtTA can rescue the mutant phenotype was determined by administering the initial dose of dox at different times after fertilization and maintaining the mothers on dox-containing water for the rest of gestation. When dox was administered at or before E9.5, the time when neural crest cells are arising from the neural tube, all $Ednrb^{rtTA}/Ednrb^{tetO}$ offspring were phenotypically wild type (Fig. 3a). As *tetO–Ednrb* mRNA was induced 9–13 hours after dox treatment (Fig. 2b), we conclude that *Ednrb* is not required before E10.

The compound heterozygotes born to females given dox at E10.5 could be divided into three phenotypic classes: (1) wild type for both melanocytes and enteric ganglia; (2) unpigmented in portions of the head and no megacolon; and (3) unpigmented head and megacolon (Fig. 3b, Table 1). The range of phenotypes could be due to several factors, including the inherent differences in development stages of the embryos within and between litters, differences in genetic background and the nonuniform distribution of dox (and therefore activation of *Ednrb*) within litters. This can be seen in Fig. 3b, where a wild-type $Ednrb^{rtTA}/Ednrb^{tetO}$ pup and a class 3 $Ednrb^{rtTA}/Ednrb^{tetO}$ pup were born in the same litter. These results indicate that the requirement for *Ednrb* signalling begins soon after E10, and that there is a temporal difference in the requirement for *Ednrb* signalling for melanocytes in the head, which are the first to arise, and those in the trunk, which develop later[12,28–31].

The litters born to females treated with dox at E11.5 produced two phenotypic groups: (1) amelanocytic, exhibiting the null *piebald-lethal* phenotype; and (2) pigmented in the caudal one- to two-thirds of the body. All progeny died of megacolon within four weeks of birth (Fig. 3c, Table 1). When dox was administered at E12.5 or later, all $Ednrb^{rtTA}/EDnrb^{tetO}$ mice exhibited the *piebald-lethal* phenotype (Fig. 3d). Thus, to rescue both the pigmentation and enteric neuron defects fully, *Ednrb* must be activated between E10 and E11.

The latest requirement for *Ednrb* function

To determine whether *Ednrb* is required later than E10, $Ednrb^{tetO}$/+ females mated to $Ednrb^{tTA}/Ednrb^{tetO}$ or $Ednrb^{tTA}$/+ males were given dox at different stages during pregnancy to repress *Ednrb* expression. When *tetO-Ednrb* mRNA was repressed by dox administration at E10.5, all $Ednrb^{tTA}/Ednrb^{tetO}$ compound heterozygotes displayed the full *piebald-lethal* phenotype (Fig. 3e). This result confirmed the

Table 1 Phenotypic analysis of *Ednrb* inducible mutants

Genotype	Duration of *Ednrb* expression	No. of animals	No. of litters	Pigmentation			Megacolon
				Wild type	Partial pigmentation	Null	
$Ednrb^{rtTA}/Ednrb^{tetO}$	E9.5–birth	4	3	4	0	0	0
	E10.5–birth	9	5	3	6 (rostral spotting)	0	4
	E11.5–birth	8	3	0	3 (rostral spotting)	5	8
	E12.5–birth	3	3	0	0	3	3
$Ednrb^{rtTA}/Ednrb^{tetO}$	0–E10.5	5	3	0	0	5	5
	0–E11.5	8	6	0	4 (caudal spotting)	4	8
	0–E12.5	3	2	3	0	0	0
	0–E13.5	1	1	1	0	0	0

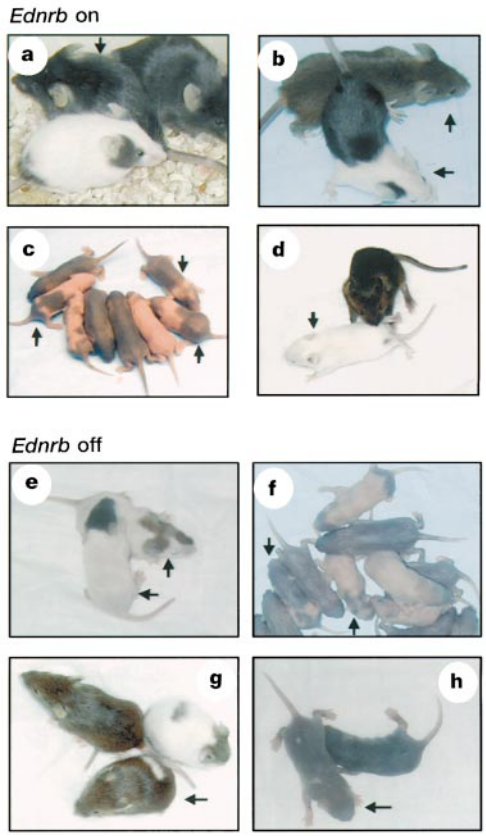

Figure 3 The temporal requirement for *Ednrb* signalling. Pregnant females were given dox at E9.5 (**a**), E10.5 (**b**), E11.5 (**c**) or E12.5 (**d**), and maintained on dox for the rest of gestation. Arrows identify all $Ednrb^{rtTA}/Ednrb^{tetO}$ heterozygotes. All other mice are *tetO/tetO*, *tetO*/+ and *rtTA*/+ litter mates. Females were given dox at E10.5 (**e**), E11.5 (**f**), E12.5 (**g**) or E13.5 (**h**), and maintained on dox for the rest of gestation. Arrows identify all $Ednrb^{tTA}/Ednrb^{tetO}$ heterozygotes. All other mice were *tetO/tetO*, *tetO*/+ and *tTA*/+ litter mates.

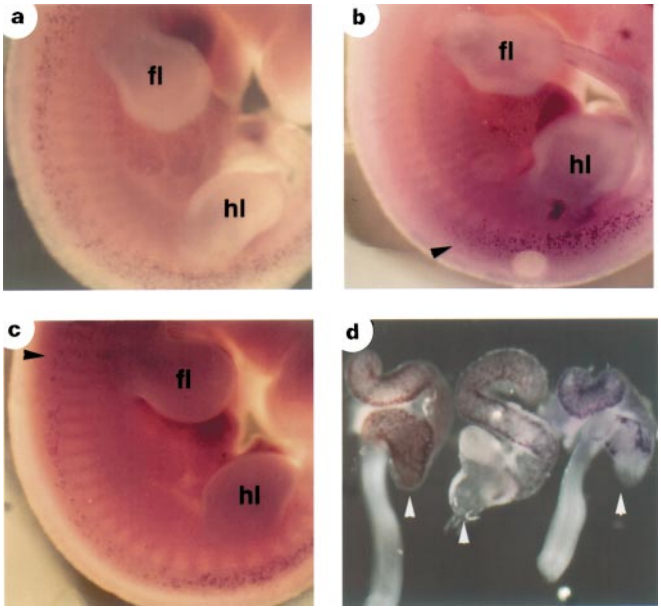

Figure 4 Detection of melanoblasts and enteric neuroblasts by *in situ* hydridization. Pregnant females were given dox at E10.5 and the embryos were removed 24 hours later and hybridized to a digoxygenin-labelled *Trp2/Dct* anti-sense probe. **a**, Wild-type; **b**, $Ednrb^{tetO}/Ednrb^{tetO}$ mutant; **c**, $Ednrb^{rtTA}/Ednrb^{tetO}$ heterozygote. fl, forelimb bud; hdl, hindlimb bud. Arrowheads indicate the most rostral melanoblasts. **d**, Pregnant females were given dox at E11.5 and the embryos were removed 24 hours later and hybridized to a *c-ret* antisense probe. The guts were dissected from wild-type (left), $Ednrb^{tTA}/Ednrb^{tetO}$ (middle) and $Ednrb^{tTA}/Ednrb^{tetO}$ (right) heterozygotes. The arrowheads indicate the position of the junction between the coecum and the colon.

conclusion drawn from Fig. 3a–d that *Ednrb* expression is required beyond E10.5. When *Ednrb* expression was inactivated after E11.5, 50% of the compound heterozygotes were *piebald-lethal*. The rest were pigmented in the rostral one- to two-thirds of the body and developed megacolon (Fig. 3f, Table 1). This reciprocal pattern of pigmentation observed in tTA-repressed versus rtTA-activated experiments is consistent with the temporal differences in melanocyte development along the rostral–caudal axis (see Discussion).

The $Ednrb^{tTA}/Ednrb^{tetO}$ compound heterozygotes obtained when *tetO–Ednrb* mRNA was turned off after E12.5 or E13.5 were wild type with the exception of one E12.5 mouse that had an unpigmented area on the lateral part of the torso, as well as a large unpigmented region on the belly (Fig. 3g, h, Table 1). None of these mice developed megacolon (Table 1). As *Ednrb* is completely repressed within 6 hours of administering dox, we conclude that *Ednrb* expression is required before E12.5 for the development of melanoblasts and enteric neuroblasts, but not at later embryonic stages.

Visualization of melanoblasts and enteric neurons

The adult phenotypes of the tet-induced *Ednrb* mice implied a rostral-to-caudal gradient in the rescue of melanoblasts between E10 and E12. To visualize this directly at the time when *Ednrb* is required, we performed *in situ* hybridization on wild-type, mutant and $Ednrb^{rtTA}/Ednrb^{tetO}$ embryos in which dox was administered at E10.5 to activate *Ednrb*. The embryos were hybridized at E11.5 to *Trp2/Dct*, one of the earliest markers of melanoblasts[29]. As shown in Fig. 4a, the wild-type embryo exhibited the expected punctate pattern of melanoblasts along the rostal–caudal axis lateral to the neural tube, whereas the mutant displayed staining only in the most caudal region of the embryo. The rescued embryo contained melanoblasts that extended further rostral than those in the mutant, but few melanoblasts were observed anterior to the forelimb buds. This is precisely the pattern one would predict from the adult phenotype (Fig. 3b).

The enteric neuroblasts were also examined using a probe to *c-ret*, an early marker for these cells[32]. As shown in Fig. 4d, the neuroblasts in the wild-type animals at E12.5 had extended beyond the coecum into the colon, whereas the neuroblasts in the mutant embryo had not proceeded past this junction (data not shown). The $Ednrb^{rtTA}/Ednrb^{tetO}$ embryos that had received dox at E11.5 displayed the mutant phenotype, with no neuroblasts beyond the coecum, very similar to $Ednrb^{tTA}/Ednrb^{tetO}$ embryos in which the gene had been repressed by dox between E11.5 and E12.5 (Fig. 4d, middle and right). This provides further evidence for a critical window for *Ednrb* signalling between E11.5 and E12.5 for enteric neuroblast development.

Discussion

We have exploited the tet-inducible system to determine when *Ednrb* gene function is required in development. By integrating the complementary tTA and rtTA components into the endogenous *Ednrb* locus, we have achieved robust expression of the *Ednrb* gene from a tet-responsive promoter. Furthermore, our studies demonstrate the utility of the tTA and rtTA transactivators for manipulating the expression of genes with tet *in vivo*.

Neural-crest-derived melanoblasts arise from the dorsal end of the neural tube around E9–E9.5 (Fig. 5). They are presumed to accumulate for 24 hours lateral to the neural tube in a region termed the migrating stage area (MSA). At E10–E10.5, these pre-migratory

melanoblasts begin dispersing dorsolaterally in a temporal rostral–caudal gradient such that by E11 the highest concentrations of migrating melanoblasts are in the cranial and sacral regions. After migrating, melanoblasts enter the epidermis between E12.5 and E13.5, where they proliferate extensively. These cells will complete their differentiation into melanocytes shortly after birth[12,28–31].

Our results indicate that *Ednrb* is required between E10 and E12.5, a period corresponding to the developmental stage where melanoblasts are migrating from the MSA (Fig. 5). The fact that the mutant phenotype could be rescued by activating *Ednrb* as late as E10 indicates that *Ednrb* may not be required for the emergence, proliferation or survival of premigratory melanoblasts (Fig. 3a, Table 1). Rather, the restricted temporal requirement for *Ednrb* indicates that its action may be required for the initiation of melanoblast migration and/or for their survival during the developmental transition from premigratory melanoblasts into migratory cells. This conclusion is consistent with the observation that DCT^{+} cells are absent in the trunk of $Ednrb^{s\text{-}l}$ mice at E10.5 (ref. 9; and Fig. 4b), as experiments have suggested that DCT is detected only in those cells that have left the MSA (in other words, only in migratory melanoblasts)[31,33]. The full rescue of the mutant phenotype in embryos expressing *Ednrb* from fertilization to E12.5 shows that *Ednrb* is not required for postmigratory epidermal proliferation, survival and differentiation (Fig. 3g, h, Table 1).

A role for *Ednrb* in the initiation of the migratory pathway is supported by the pigmentation patterns we observed in mice where *Ednrb* was activated or repressed between E10.5 and E11.5. The rescue of caudal but not rostral cells in some $Ednrb^{rtTA}/Ednrb^{tetO}$ compound heterozygotes that developed until E10.5 or E11.5 in the absence of dox indicates that caudal melanoblasts require *Ednrb* function later than do the rostral cells. This result is consistent with the observation that caudal cells emerge later than rostral cells (Figs 3b, c and 4c). The absence of the most rostral pigment cells could be due to the loss of melanoblasts in the MSA, which would explain why later *Ednrb* expression fails to rescue the cells, and/or the inability of the MSA melanoblasts to enter the migratory pathway at later stages, possibly because the environment is no longer permissive. In the reciprocal experiment, where *Ednrb* is turned off at E11.5, rostral cells were rescued more efficiently than caudal cells (Fig. 5b, Table 1). This pattern is consistent with rostral melanoblasts having initiated their migration before the cessation of *Ednrb* expression, whereas caudal cells were still in the MSA. Rostral melanoblasts in the epidermis have been observed as early as E11 (ref. 34).

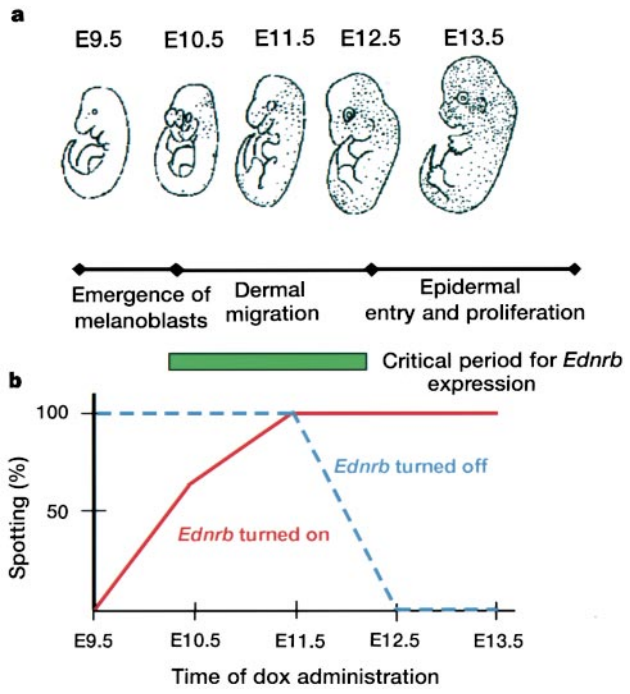

Figure 5 *Ednrb* signalling is required during melanoblast migration. **a**, The appearance of c-kit+ and TRP2+ melanoblasts in mouse embryos at different stages of embryogenesis is adapted from Yoshida *et al.*[12]. **b**, The degree of white spotting in $Ednrb^{rtTA}/Ednrb^{tetO}$ heterozygotes (blue) and $Ednrb^{rtTA}/Ednrb^{tetO}$ heterozygotes (red) treated with dox for the times indicated. The critical period when *Ednrb* signalling is required is indicated by the green box.

Finally, these studies show that *Ednrb* is also required between E10.5 and E12.5 for the development of enteric neuroblasts (Table 1). This conclusion is supported by observations using a *lacZ*-tagged *Ednrb* null allele which showed that the progress of migrating enteric neuroblasts is delayed by E11 in mutant embryos (M.K.S., unpublished data). Likewise, the migration of the enteric neurons is displayed in *Ednrb*-null *spotting lethal* rats[35]. However, our observations differ from previous studies that concluded that differences between wild-type and mutant mice are not observed until E12 (ref. 36). The discrepancy may be due to differences in the genetic backgrounds or to the fact that the earlier study used transgenic mice marked with a *dopamine-β-hydroxylase lacZ* gene to identify enteric neuroblasts and this marker may not be expressed in all *Ednrb*-expressing cells. On the other hand, our results are consistent with a model[37,38] proposing that EDN3 may be required to maintain a sufficient pool of enteric neuronal precursors in a migratory undifferentiated state. According to this model, in the absence of *Ednrb* signalling migrating enteric neuroblasts differentiate prematurely and become postmitotic, thereby failing to colonize the lower bowel. The finding that *Edn3* is expressed at the highest levels in the coecum is consistent with this model[39]. Thus it could be argued that our study has uncovered a unifying logic for the function of *Ednrb* in regulating migration of both melanoblasts and enteric neuroblasts.

Despite our successful use of the tet-inducible system, we note several limitations. First, the level of *tetO–Ednrb* mRNA in the absence of dox in tTA animals or in the presence of dox in rtTA mice, although sufficient to rescue the mutant phenotype, is lower than that of endogenous *Ednrb* mRNA in heterozygotes (~60%; Fig. 2). By improving splicing and codon usage, the production of tTA and rtTA have been greatly improved in mammalian cells[23]. Second, although the *tetO* promoter was repressed efficiently in *rtTA* mice in the absence of dox, or in *tTA* mice in the presence of the drug (Fig. 2), this was not the case when we deleted the *Pgk1–hygro* cassette from the responder line. Instead, we obtained partially pigmented $Ednrb^{rtTA}/Ednrb^{tetO}$ mice that resembled the hypomorphic $Ednrb^{s}$ allele[3] in the absence of dox (data not shown). Thus, it appears that the *Pgk-1* promoter fortuitously inhibited the basal *tetO* promoter.

Despite these limitations, the integration of the responder and activators into the endogenous locus by homologous recombination alleviated many problems associated with previous studies using transgenic mice. The ability of dox to cross the placenta and rapidly regulate gene expression *in utero*, coupled with the considerable flexibility that the tet system affords to investigators, makes this system very useful for studying mammalian development. The *tTA* and *rtTA* mice that we generated will be generally useful for studying other genes implicated in melanocyte and enteric neuron development, as well as genes that function in other lineages where *Ednrb* is expressed. □

Methods

Targeting vectors and embryonic stem cells

Ednrb genomic DNA was isolated from a 129Sv/J λFIXII library (Stratagene). For the transactivator constructs, the forward primer 5′-CTGCAGCACCATGTCTAGAT TAGA-TAAAAGT-3′ and the reverse primer 5′-GGTACCGCCACCTTTGTTCATGG CAGC-3′ were used to PCR amplify the tTA and rtTA cassettes from plasmids pUHG15-1 (ref. 18)

and pUHG17-1 (ref. 17), respectively. The targeting vector contained PGK-neo^R flanked by two *loxP* sites, a 3.3-kb genomic fragment that contains 200 bp of the untranslated region but not the *Ednrb* ATG initiation codon and a 6-kb *Bg*II–*Eco*RV 3′ flanking fragment. These constructs were transfected into E14.1 embryonic stem (ES) cells[40], and positive clones were selected with 200 μg ml^{-1} active G418 as described[41]. For the responder construct, a 1.4-kb *Ednrb* cDNA containing the entire coding region of the protein[26] was subcloned downstream of the tetO promoter of pUHG10-3 (a gift from L. Hennighausen). The targeting vector contained PGK-hygro^R flanked by *loxP* sites, a 2.7-kb *Kpn*I–*Stu*I 5′ genomic fragment and a 6-kb *Bg*II–*Eco*RV 3′ genomic fragment. The targeting vector was transfected into CJ7 ES cells[42], and positive clones were selected with 150 μg ml^{-1} active hygromycin as described[43]. ES cells were injected into C57BL/6J blastocysts and germline mice were generated as described[43].

Genotyping *Ednrb* alleles

The three targeted *Ednrb* alleles were distinguished by PCR of tail or yolk sac DNA using the following primers: Ednrb5′, 5′-CCAGACTGAAAACAGCAGAGCGGC-3′; Ednrb 2, 5′-GGTCTCCCAGAGCCAGACTGGCGATC-3′; CMV407, 5′-GGCGTGTACGGTGG-GAGG-3′; tTA416, 5′-GCATTAGAGCTGCTTAATGAGG-3′; tTA415, 5′-TCTTGATCTTCCAATACGCAACC-3′. The Ednrb5′ and Ednrb2 primers produce a 495-bp wild-type *Ednrb* exon1 fragment. The CMV407 (ref. 18) and Ednrb2 primers identify a 610-bp *tetO*–*Ednrb* fragment. Primers tTA416 and tTA415 amplify 410-bp tTA or rtTA fragments. These alleles were further distinguished by *Nru*I and *Hind*III restriction fragment length polymorphisms within the PCR products. The PCR reactions were performed under the following conditions: 94 °C for 30 s, 57 °C for 45 s, 72 °C for 60 s for 35 cycles.

The PGK–neo cassette was deleted from *Ednrb*^{rtTA}/+ and *Ednrb*^{rtTA}/+ mice by mating heterozygous males from each line to EIIa-cre females as described[25].

Doxycycline administration

For timed pregnancies, the morning in which the vaginal plug was observed was E0.5. An initial dose of 200 μl of 1 mg ml^{-1} dox (Sigma) was administered to pregnant females by oral gavage between 9 and 10 AM. Subsequently, the females were maintained on 1 mg ml^{-1} dox water for the duration of the pregnancies. The megacolon phenotype was determined by observing a gross distension of the colon post-mortem in affected animals as described[5].

RNA analysis

Total RNA was isolated from E11–E12 embryos by Trizol extraction (Gibco-BRL). PolyA+ RNA was isolated by the Micro FastTrack Kit (Invitrogen) and analysed by agarose gel electrophoresis and northern blotting[26].

In situ hybridization

Whole-mount *in situ* hybridization was performed on embryos as described[44]. To detect melanoblasts, a *Trp2/Dct*-specific riboprobe[29] was hybridized to *Ednrb*^{rtTA}/*Ednrb*^{tetO} E11.5 embryos that had been given dox at E10.5. To detect enteric neuroblasts, a *c-Ret*-specific riboprobe[32] was hybridized to *Ednrb*^{rtTA}/*Ednrb*^{tetO} and *Ednrb*^{tTA}/*Ednrb*^{tetO} E12.5 embryos that had been given dox at E11.5.

Received 15 June; accepted 12 October 1999.

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Acknowledgements

We thank L. Henninghausen and H. Bujard for providing constructs; H. Westphal for providing EIIa-cre transgenic mice; the members of our laboratory for helpful discussions; and M. Cleary for critical comments on the manuscript. M.K.S. was a Jane Coffin Childs Postdoctoral Fellow and S.M.T. is an Investigator of the Howard Hughes Medical Institute. R.S.I. isolated and characterized the *Ednrb* genomic clones and J.M.L. injected the targeted ES cells into blastocysts. M.K.S. and S.M.T. were responsible for the design of the experiment and the writing of the manuscript, and M.K.S. for the generation of the targeting vectors, targeting of ES cells and analysing the mice.

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